

**DIAGNOSTICS AND THERAPEUTICS FOR AUTOSOMAL DOMINANT  
HEMOCHROMATOSIS**

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BACKGROUND OF THE INVENTION

Field of the Invention

[0001] The present invention relates generally to the gene,  
and mutations, that are responsible for the disease  
hemochromatosis (MIM604653). In particular, the present  
invention provides for the presence of one or more  
mutations on the SLC11A3 gene which results in aberrant  
SLC11A3 mediated iron transport. The invention also  
relates to methods for screening for HH and to HH  
diagnosis, prenatal screening and diagnosis, and  
therapies of HH disease, including gene therapeutics,  
protein and antibody based therapeutics, and small  
molecule therapeutics. The invention further relates to  
drugs and therapies developed for the treatment of  
patients with HH or anemia.

Description of the Related Art

[0002] Over the years, several known genes involved in iron  
metabolism have been implicated in the pathology of HH.  
However, not all instances of HH patients can be  
explained by mutations in these genes. In particular, it  
is known that approximately 60-85% of all instances of HH

in adult patients are indicated by homozygosity for the C282Y mutation in the HLA-H/HFE gene on Chromosome 6p. Compound heterozygosity accounts for an additional 10% of cases. It is also known that a form of juvenile hemochromatosis maps to chromosome 1q and that a single family was found with a mutation in a transferrin receptor gene (TFR2) on chromosome 7q. However, the remaining 5-15% of patients indicated with HH do not possess any mutations on the known genes. For example, Kato et al., describe a heterozygous A49T mutation in the 5'UTR of the H-subunit of ferritin without specifically delineating the contributions of this gene to the HH disease state (Am. J. Human Genetics 69: 191-7 (2001)). Clearly, neither the precise physiological mechanism of iron overaccumulation nor every gene which is defective in this disease has been described.

[0003] Hemochromatosis is an inherited disorder of iron metabolism wherein the body accumulates excess iron. In symptomatic individuals, excess iron is deposited in a variety of organs which leads to organ failure. Disease states such as cirrhosis, diabetes, sterility, and other serious illnesses occur as a result. It has also been discovered that HH can be inherited by a dominant or

pseudo-dominant mode of inheritance. Heretofore, HH was believed to be inherited solely as a recessive trait. In particular, the prior art limits HH to patients having homozygotes carrying two defective copies of the gene.

5 [0004] Hemochromatosis is also one of the most common genetic disorders. The prior art estimates that approximately 10% of individuals of Western European descent carry one copy of the HH gene mutation and that there are about one million homozygotes in the United States. HH, thus, represents one of the most common genetic disease mutations in individuals of Western European descent. Although HH ultimately produces debilitating symptoms, the majority of carriers have not been diagnosed. Indeed, it has been estimated that no more than a small fraction of affected individuals in the United States have been diagnosed with this condition.

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20 [0005] Current diagnostic methods fail to comprehensively test for HH in individuals who are at risk, especially those individuals who are presymptomatic. Although blood iron parameters can be used as a screening tool, a confirmed diagnosis often employs a costly, invasive and high risk liver biopsy. An additional problem is that symptoms of HH are similar to those of other conditions

and the severe effects of the disease tend not to appear immediately. Thus, there is a clear need for the development of an inexpensive and noninvasive comprehensive diagnostic test for detection of HH in order to facilitate comprehensive diagnosis and to provide comprehensive presymptomatic detection for the identification of HH carriers. Accordingly, comprehensive methods to identify persons who may be destined to become symptomatic in order to intervene in time to prevent excessive tissue damage associated with iron overload are desirable.

[0006] U.S. Patent No. 6,025,130 ("Thomas et al.") describes a gene indicated for HH in the HLA region of Chromosome 6. Although Thomas et al. discloses a gene thought to cause HH, it does not provide for the remaining population of individuals who are symptomatic of HH but do not carry the mutation as taught by Thomas et al.

[0007] In the search for additional genes indicated for HH, one study generally concluded that HH can occur in adults who do not have pathogenic mutations in the hemochromatosis gene. See *N Engl J Med* 341:725-32 (1999). In particular, the study found that members of

a single family of Italian descent displayed symptoms of one or more distinct genetic diseases that cause a type of adult hereditary iron overload which was not associated with the known HH gene. The study, however, failed to provide for the gene hypothesized to cause the disease.

[0008] Another study relating to Zebrafish concluded that the gene ferroportin 1 (FNPI) may be involved in mammalian disorders of iron deficiency or overload. *Nature*, 403:17 (2000). In particular, the study focused on hypochromia caused by inadequate circulatory iron levels in embryonic Zebrafish. The study found that two independent autosomal recessive mutations caused hypochromia in the Zebrafish embryo. Mammalian homologues of these genes include SLC11A3.

[0009] However, the transversion site disclosed by the study differs from the present invention and is not believed to be responsible for HH. In particular, the study specifically teaches that a C-to-A transversion at codon 361 prematurely terminates translation of the SLC11A3 gene and that a G-to-T transversion at an undisclosed location results in a single amino acid change from Leucine to Phenylalanine. Additionally, the

study fails to explain how the amino acid change affects the SLC11A3 protein. Accordingly, the study does not provide any motivation or suggestion to one of ordinary skill in the art to believe that a C-to-A transversion at codon 361 or a G-to-T transversion at an undisclosed location causes about 5-15% of all HH cases.

[0010] Genetic markers are also known for a mutation in recessive hemochromatosis. Diagnostic methods and kits for its determination are disclosed by U.S. Patent No. 5,753,438 and U.S. Patent No. 5,705,343.

[0011] U.S. Patent No. 5,712,098 ("Tsuchibashi et al.") also discloses recessive hereditary hemochromatosis diagnostic markers and diagnostic methods for same.

[0012] U.S. Patent No. 5,674,681 ("Rothenberg") discloses a method for identifying an individual having or predisposed to having hemochromatosis by detecting a mutation in the gene encoding a nonclassical MHC class I heavy chain. Rothenberg also discloses methods for treating recessive hemochromatosis involving administration of a "leczyme" having similar specificity for a carbohydrate ligand as the leczyme involved in the disease state. Clearly, the prior art fails to provide for the remaining 5-15% of patients indicated with HH who

do not possess any mutations of known genes.

[0013] Accordingly, it would be highly desirable to identify, isolate, clone, and sequence the gene responsible for the remaining 5-15% of patients indicated with HH who do not possess any mutations of known genes. Such identification, isolation, cloning, and sequencing of the gene would enable the design and manufacture of products useful for the diagnosis and screening for HH. Identification of individuals affected with HH will allow initiation of this therapy, which can prevent symptoms, arrest progression of organ damage, and in some cases reverse pathology due to iron overload.

[0014] Moreover, such identification, isolation, and cloning of the gene would enable the study of the operation of the gene in the development of iron overload diseases, in general, and HH in particular. Further, it would be highly desirable to provide therapeutics for iron overload diseases, and HH disease in particular, as well as oxidative free radical diseases, reactions, and processes in general. The identification, isolation, sequencing, and cloning of the gene responsible for the remaining 5-15% of patients indicated with HH who do not possess any mutations of known genes, and identification



of its protein products would also facilitate improved therapeutic development.

[0015] These and other embodiments of the invention will be apparent from the detailed description and the claims.

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SUMMARY OF THE INVENTION

[0016] The present inventive subject matter relates to an identified, isolated, cloned and sequenced mutated SLC11A3 gene responsible for HH in about 5-15% of patients indicated with HH who do not possess any mutations of known genes thought to cause HH.

[0017] The principal mutation found in the gene comprises a single nucleotide substitution of A-to-C at positions 734 (A734C) in exon 5 which causes a significant amino acid change in the protein product expressed by the gene. One such amino acid change that may cause disruption of normal regulation of SLC11A3 activity as a result is the amino acid substitution of asparagine by histidine at position 144 (N144H).

[0018] The present invention, therefore, represents the first opportunity to accurately and noninvasively screen and diagnose HH in the 5-15% of patients indicated with HH who do not possess any mutations of known genes

thought to cause HH. In addition, the present invention enables the study of the SLC11A3 gene. Through such study, the development of therapeutics (gene, protein replacement, antibodies, small molecules, and the like) for HH disease will be enabled.

[0019] In accordance with a first embodiment of the present invention, there is provided an isolated nucleic acid comprising a nucleic acid sequence corresponding to the nucleic acid sequence of SEQ ID NO. 1.

[0020] Another embodiment of the present inventive subject matter is a method for diagnosing a patient as having an increased risk of developing HH disease, comprising the steps of providing DNA or RNA from the individual; and assessing the DNA or RNA for the presence or absence of a base mutation at position 734 (A734C) in exon 5 of the SLC11A3 gene wherein the absence of the base mutation indicates the absence of a HH gene mutation in the genome of the individual and the presence of the base mutation indicates the presence of the HH gene mutation in the genome of the individual and an increase risk of developing HH disease.

[0021] Yet another embodiment of the present inventive subject matter is an oligonucleotide in a range of 8-18

consecutive nucleotides selected from a sequence unique to SEQ ID NO. 1 or the complement of SEQ ID NO. 1.

[0022] Another embodiment of the present inventive subject matter is a isolated polypeptide comprising the amino acid sequence as shown in SEQ ID NO. 2, SEQ ID NO. 3, or SEQ ID NO. 4, wherein SEQ ID NO. 2, SEQ ID NO. 3, and SEQ ID NO. 4 are the normal peptide product of the SLC11A3 gene, the mutated peptide product of the SLC11A3 gene and the wild type peptide product of the SLC11A3 gene, respectively.

[0023] Yet another embodiment of the inventive subject matter is a pharmaceutical composition comprising the isolated polypeptide as shown in SEQ ID NO. 2.

[0024] Another embodiment of the present inventive subject matter is a kit for the detection of the presence or absence of a base mutation at position 734 (A734C) of the SLC11A3 gene. In another embodiment, the kit further comprises oligonucleotide primers for amplifying the DNA containing the base-pair polymorphisms.

[0025] Yet another embodiment of the present inventive subject matter is a genetic marker predictive of a hereditary hemochromatosis (HH) gene mutation comprising a partial sequence of SEQ ID NO. 1 and sequences

complementary therewith. In one aspect, the nucleic acid is DNA. In another aspect, the DNA is cDNA. In another aspect, the nucleic acid is RNA. In another aspect, the nucleic acid is a nucleic acid sequence corresponding to the nucleic acid sequence of SEQ ID NO:1.

[0026] Still yet another embodiment of the present inventive subject matter of the invention is a cloning vector comprising a coding sequence of a nucleic acid as set forth above and a replicon operative in a host cell for the vector.

[0027] Further still yet another embodiment of the present inventive subject matter is an expression vector comprising a coding sequence of a nucleic acid set forth above operably linked with a promoter sequence capable of directing expression of the coding sequence in host cells for the vector.

[0028] Another embodiment of the present inventive subject matter is host cells transformed with a vector as set forth above.

[0029] Yet another embodiment of the present inventive subject matter is a method of producing a mutant SLC11A3 polypeptide comprising: transforming host cells with a vector capable of expressing a polypeptide from a nucleic

acid sequence as set forth above; culturing the cells under conditions suitable for production of the polypeptide; and recovering the polypeptide.

[0030] Still yet another embodiment of the present inventive subject matter is a peptide product consisting of a polypeptide having the amino acid sequence corresponding to the sequence of SEQ ID NO. 2, SEQ ID NO. 3, or SEQ ID NO. 4. In one aspect, the peptide is labeled. In another aspect, the peptide is a fusion protein.

[0031] Further still yet another embodiment of the present inventive subject matter of the invention is a use of a peptide as set forth above as an immunogen for the production of antibodies. In one embodiment, there is provided an antibody produced in such application. In one embodiment, the antibody is labeled. In another embodiment, the antibody is bound to a solid support. In a further embodiment, the antibody is monoclonal. Another embodiment of the present inventive subject matter is an oligonucleotide primer useful for amplification of DNA, the oligonucleotide primer designed on the basis of the DNA sequence of any one of SEQ ID NO: 1.

[0032] Yet another embodiment of the present inventive subject matter is a method for diagnosing whether a patient is afflicted with hereditary hemochromatosis (HH) disease, comprising: contacting cells of the patient with antibodies directed against an epitope on an SLC11A3 protein product corresponding substantially to SEQ ID NO. 3, or SEQ ID NO. 4; and observing whether the antibodies localize on the cells. In one embodiment, the method is conducted *in vitro*. In another embodiment, the method is conducted *in vivo*.

[0033] Still yet another embodiment of the present inventive subject matter is a method for treating a patient diagnosed as having hereditary hemochromatosis (HH) disease, comprising delivering a polypeptide corresponding to the amino acid sequence of SEQ ID NO:2 to tissues of the patient. In one aspect, the polypeptide is delivered directly to the tissues. In another aspect, the polypeptide is delivered intravenously. In another embodiment, the polypeptide is delivered to the tissues through gene therapy.

[0034] Further still yet another embodiment of the present inventive subject matter is an animal model for hereditary hemochromatosis (HH) disease, comprising a

mammal possessing a mutant or knocked-out SLC11A3 gene.

[0035] Another embodiment of the present inventive subject matter is metal chelation agents derived from nucleic acid sequences described above or from a peptide product as described above in a physiologically acceptable carrier. In one embodiment, the metal is selected from the group consisting of iron, mercury, cadmium, lead, and zinc.

[0036] Yet another embodiment of the present inventive subject matter is a method to screen mammals for susceptibility to metal toxicities, comprising, screening such mammals for a mutation in the SLC11A3 gene and wherein those mammals identified as having a mutation are more susceptible to metal toxicities than mammals not identified as having a mutation. In one aspect, the metal is selected from the group consisting of iron, mercury, cadmium, lead, and zinc.

[0037] Still yet another embodiment of the present inventive subject matter is a method for selecting patients infected with hepatitis virus for  $\alpha$ -interferon treatment, comprising screening such patients for a mutation in the HH gene and wherein those patients not identified as having a mutation are selected to proceed

with  $\alpha$ -interferon treatment and those identified as having a mutation are selected to undergo phlebotomy prior to  $\alpha$ -interferon treatment.

[0038] Further still yet another embodiment of the present inventive subject matter is a T-cell differentiation factor comprising a moiety selected from the group consisting of molecules derived from nucleic acid sequences described above and from peptide products described above.

[0039] Another embodiment of the present inventive subject matter is a method for screening potential therapeutic agents for activity in connection with HH disease, comprising: providing a screening tool selected from the group consisting of a cell line, a cell free, and a mammal containing or expressing a defective SLC11A3 gene or gene product; contacting the screening tool with the potential therapeutic agent; and assaying the screening tool for an activity selected from the group consisting of SLC11A3 protein folding, iron uptake, iron transport, iron metabolism, receptor-like activities, upstream processes, downstream processes, gene transcription, and signaling events.

[0040] Yet another embodiment of the present inventive



subject matter is a therapeutic agent for the mitigation of injury due to oxidative processes *in vivo*, comprising a moiety selected from the group consisting of molecules derived from nucleic acid sequences described above and from peptide products described above.

[0041] Still yet another embodiment of the present inventive subject matter is a method for diagnosing whether a patient is afflicted with anemia, comprising: contacting cells of the patient with antibodies directed against an epitope on an SLC11A3 protein product corresponding substantially to SEQ ID NO. 3, or SEQ ID NO. 4; and observing whether the antibodies localize on the cells. In one embodiment, the method is conducted *in vitro*. In another embodiment, the method is conducted *in vivo*.

[0042] Further still yet another embodiment of the present invention involves a kit for detecting or identifying HH or anaemia in a sample, comprising a means for collecting a sample of breast epithelial cells and a means for detecting an A-to-C transversion at position 734 (A734C) in the SLC11A3 gene.

[0043] Yet another embodiment of the present invention is directed to a pharmaceutical composition for treating

iron overload or anaemia in an animal, comprising a therapeutically effective amount of an catalytically active fragment of an amino acid sequence as shown in SEQ ID No. 2, SEQ ID NO. 3 or SEQ ID NO. 4, and a pharmaceutically acceptable carrier.

**BRIEF DESCRIPTION OF THE DRAWINGS**

[0044] The invention may be better understood by reference to the drawings wherein:

[0045] Figure 1 is a pedigree of Dutch autosomal dominant hemochromatosis family with haplotypes for chromosome 2q markers (ordered from centromere to telomere). Blackened symbols represent affected individuals. White symbols represent unaffected individuals. Partly filled symbols represent possibly affected individuals. "+" indicates confirmed carriers of the N114H mutation. Bars next to haplotypes indicate a shared risk haplotype.

[0046] Figure 2 is a genomic structure of SLC11A3 spanning 20 kb. Exons are indicated by boxes. 5' and 3' untranslated regions are indicated by the white boxes in exons 1 and 8. An iron responsive element is located in the 5' region of exon 1. Exon number and sizes are indicated above the boxes and intron sizes are indicated

below the boxes. Flanking polymorphic markers are indicated but not placed according to scale. The A-to-C transversion at the (A734) position leading to an asparagine substitution by histidine at position 144 (N144H) is indicated by an arrow.

[0047] Figure 3 is a mutation analysis wherein the control (left) and patient (right) sequences are shown, depicting the heterozygous mutation (M). Amino acid sequences are indicated above the corresponding codons.

[0048] Figure 4 is a sequence homology analysis of the region around the mutation. The N144H amino acid substitution is indicated by an arrow. Predicted transmembrane domains are indicated by a border surrounding the amino acid sequence.

[0049] Figure 5 is a SLC11A3 expression in human tissues wherein the left panel is a Northern blot of adult human tissues; the middle panel of the human digestive tract; and the right panel is of human monocytes and lymphoblast cells.

[0050] Figure 6 is a model for iron transport from the gut lumen to body through enterocytes into the circulation and subsequent accumulation in the liver.

**DETAILED DESCRIPTION OF THE INVENTION**

[0051] As used herein, "marker" refers to a DNA sequence polymorphism flanked by unique regions. These regions flanking the "marker" can be utilized for the design and construction of oligonucleotides for amplifying the relevant DNA portions and detecting the polymorphisms therein.

[0052] The term "HH disease" refers to hereditary hemochromatosis disease. The criteria utilized herein to assess whether a patient is affected with the HH disease (i.e., whether the patient is an "affected individual" having "affected chromosomes") has been established by the diagnostic criteria set out in Crawford et al. *Am J Hum Genet* 57:362-367 (1995).

[0053] The term "lod score" refers to a statistic that is used to detect genetic linkage and that is equal to the logarithm to base 10 of the ratio of the probability that the data in a linkage experiment would be obtained if the genetic loci under investigation were linked to the probability that the data would be obtained if the loci were not linked.

## A. Discovery of the SLC11A3 Gene Mutation

[0054] A large Dutch family was used wherein hemochromatosis segregates as a dominant trait among family members (Fig. 1). The clinical symptoms in patients from this family were similar to complaints in other HH patients and include joint pains, osteoarthritis, fatigue, cardiomyopathies, endocrine disorders such as diabetes mellitus. For several markers on chromosome 2q, two point linkage analyses yielded positive lod scores (Table 1).

Table 1

Marker	Recombination fraction ( $\theta$ )						
	0	.01	.05	.10	.20	.30	.40
D2S335	0.77	1.36	1.75	1.74	1.40	0.89	0.32
D2S2314	1.90	1.87	1.72	1.54	1.14	0.71	0.27
D2S2273	0.69	1.13	1.50	1.51	1.22	0.78	0.29
D2S389		2.96	2.73	2.44	1.80	1.09	0.38
D2S2167	0.45	0.44	0.38	0.31	0.21	0.13	0.07
D2S117	1.60	1.64	1.70	1.62	1.27	0.78	0.27
D2S311	0.99	1.04	1.14	1.13	0.91	0.55	0.16
D2S2392	1.59	1.55	1.41	1.23	0.86	0.48	0.17
D2S2289	1.06	1.11	1.18	1.15	0.91	0.57	0.22
D2S325	0.90	0.90	0.94	0.95	0.81	0.52	0.19
D2S2382	1.12	1.09	1.00	0.87	0.61	0.36	0.13

[0055] Patients were suspected to have HH if they had a blood ferritin level above 450 ng/ml and/or transferrin saturation >50%. This diagnosis was further confirmed by a specialist through liver biopsy and Magnetic Resonance Imaging (MRI). Diseases with similar expression such as Hereditary Hyperferritinemia Cataract Syndrome (HHCS) and aceruloplasemia were excluded. Patients were treated by putting them on a regular phlebotomy regime.

[0056] Genomic DNA was isolated from peripheral blood as described. For the systematic genome scan short tandem repeat polymorphisms (STRPs) from the Genethon markers set were used. Genomic DNA (25 ng) was amplified in 10 ml PCR reactions containing 1X GeneAmp PCR Gold Buffer; 1.5 mM MgCl<sub>2</sub>; 25 ng of fluorescent forward primer; 25 ng unlabelled reverse primer and 0.4 units of AmpliTaq Gold DNA polymerase (Applied Biosystems). Initial denaturation was 15' at 95°C followed by 32 cycles of 30'' denaturation at 95°C, 30'' annealing at 55°C and 90'' extension at 72°C. PCR products were pooled and loaded on an ABI377 automated sequencer (filterset D; 5% denaturing FMC LongRanger acrylamide gel), data were analyzed using ABI GeneScan3.1 and ABI Genotyper2.1 software.

[0057]           Simulation studies using the SLINK program showed that the family was sufficient for finding significant evidence for linkage with an average maximum lod score of  $Z=3.24$  by using the following linkage analysis model: HH was assumed to be a autosomal dominant disorder with a disease gene frequency of 0.001. To account for differences in age at onset of male and female patients three (age and sex dependent) liability classes were defined with penetrances of 0.02, 0.6 and 0.8 for class 1 (male < 30, female < 40 years), class 2 (male 30-60 and female 40-70 years) and class 3 (male >60, female >70 years), respectively. Two point linkage analysis was performed using the MLINK and ILINK programs of the LINKAGE package (version 5.1). Recombination frequency (q) was assumed to be equal for males and females. Equal allele frequencies were used. For haplotype analyses, phase was assigned on the basis of the minimum number of recombination events.

[0058]           The highest lod score was obtained with marker D2S389, ( $Z_{\max} = 3.01$  at  $q = 0.0$ ). Additional markers were tested from the region and haplotypes constructed therefrom (Fig. 1). Accordingly, it was found that all patients share a common allele for markers D2S389 and

D2S2167. However, recombination events were identified for D2S2273 on the centromeric site and D2S117 on the telomeric site. These sites define the critical region to a maximum of 25 cM on the sex average genetic map. This is the fourth locus identified for HH and is named HFE4.

[0059] Based on the lod scores, a search for candidate genes in public databases and literature identified a large number of expressed sequence tags and genes with already known function. Of these choices, SLC11A3 was the most likely. SLC11A3 also known as IREG1, MTP1, and FPN1 has been mapped to human chromosome 2q.

B. Characterization and Expression  
of SLC11A3 gene

1. Sequence

[0060] The human SLC11A3 gene encompasses 20 kb and consists of 8 exons. The open reading frame of 1716 bp starts at position 305 of exon 1 and ends at position 314 of exon 8 (Fig. 2) encoding a protein of 571 amino acids with 9 or 10 transmembrane domains.

[0061] Although conflicting evidence exists in the prior art, it has been confirmed that human SLC11A3 is expressed in most tissues but especially in those tissues



involved with iron metabolism. Accordingly, expression of SLC11A3 is highest in the digestive tract, liver, placenta, kidneys and monocytes (Fig. 5).

[0062] SLC11A3 is also a basolateral membrane protein implicated in the movement of iron across the enterocytes into the circulatory system. In particular, SLC11A3 mRNA contains in its 5' untranslated region a stem-loop structure characteristic of an iron responsive element (IRE). This IRE binds to iron responsive proteins 1 and 2 (IRP1 and IRP2) indicating that expression of SLC11A3 is probably regulated by intracellular iron levels.

[0063] Moreover, in all symptomatic HH patients, all exons of the mutated SLC11A3 gene including intron-exon boundaries contain a heterozygous A-to-C transversion at position 734 (A734C). Simple statistical analysis compared to 200 healthy controls from the Dutch general population suggests that the heterozygous A-to-C transversion at position 734 (A734C) base change is the causative mutation for HH.

[0064] The mutation leads to an amino acid substitution of asparagine by histidine at position 144 (N144H). Linkage disequilibrium of this mutation with another yet unidentified mutation is possible but this is not likely

for a number of reasons. First, no other sequence alteration segregating with the disease was detected. Second, due to the highly conserved nature of asparagine in vertebrates (Fig. 4), the substitution of asparagine suggests a pronounced effect on SLC11A3 function.

[0065] Several possible results arise from the mutation. Protein structure prediction programs predict that the mutation is expressed in a transmembrane domain which may explain the effect of the mutation on the SLC11A3 structure. Another result may arise from the fact that asparagine is a neutral amino acid, i.e. when asparagine is substituted with the polar histidine the hydrophobicity of the transmembrane domain may induce protein folding. Finally, it is clear that the mutation is important for metal ion binding because it resides within a region of the protein that contains other divalent metal transporters that otherwise show little homology to SLC11A3 (Fig. 4).

## 2. Structure/Function of SLC11A3 and mutated SLC11A3 gene product

[0066] Ferrous iron ( $\text{Fe}^{2+}$ ) is transported through the apical surface of the enterocytes by the divalent metal transporter (DMT1) wherein a model for iron transport is

depicted in Fig. 6. Iron can then be stored as ferritin or transported across the basolateral membrane by SLC11A3 with the aid of hephaestin (Hp). In blood plasma, iron is then loaded onto ferrotransferrin as ferric iron (Fe<sup>3+</sup>) and subsequently taken up by body tissues such as the liver by transport over a transferrin receptor (TFR2) that is associated with HFE.

[0067] Mutated SLC11A3 in HFE4 patients might cause disruption of normal regulation of SLC11A3 activity and lead to transport of excessive amounts of iron out of enterocytes towards the circulation suggesting that the N144H mutation in SLC11A3 is an activating mutation. As a result iron levels in enterocytes are expected to be depleted.

[0068] Activity of DMT1 is controlled by intracellular iron levels. Low iron levels will lead to increased iron uptake by DMT1. The excessive uptake of iron from the lumen of the gut and transport of iron to the circulation then leads to accumulation of iron in blood plasma and body tissues. The mutation results in overactivation of SLC11A3.

[0069] Thus described, a heterozygous mutation in the SLC11A3 gene is disclosed that explains autosomal

dominant hemochromatosis and which may also have important implications for explaining hemochromatosis in the 5-15% of patients who do not carry mutations in the HH and TFR2 genes. Moreover, SLC11A3 is a target for developing therapeutic approaches for anemia as described herein because the activating mutations in SLC11A3 might result indirectly in activation of DMT1 activity.

3. Mutation analysis

[0070] SLC11A3 has been described under several aliases such as iron regulated transporter gene 1 (IREG1), metal transport protein 1 (MTP1) or solute carrier family 11 member 3 (SLC11A3). (GENBANK accession numbers AF226614, AF231121 and AF215636).

[0071] The genomic structure of SLC11A3 was determined by aligning cDNA (AF231121) and genomic (RP11-270G18, AC013439) sequences. SLC11A3 exons (1-8) were amplified from genomic DNA using primers designed to flank intronic sequence (at least 50 bp intronic sequence on both sides of each exon).

The following primers were used:

[0072] Exon 1: Forward primer 5'-3' (F-)  
CCCCGACTCGGTATAAGAG, reverse primer 5'-3' (R-)

TTCCTCCAGAACTCG TGT AG;

[0073] Exon 2: F-TGGATAAGCATTCTGCCCTC, R-  
TAAAGCATGTGTACTTGGATG;

[0074] Exon 3: F-AATGTAGCCAGGAAGTGCC, R-  
AGAGGTGGTGCCATCTAAG;

[0075] Exon 4: F-GGATAAGAACAGTCTCACTG, R-  
TTCATCCTTTACCACTACCAG;

[0076] Exon 5: F-TTAAA CTGCCTTGTTTAGTG, R-  
GCCTCATTATCACCACCG;

[0077] Exon 6: F-TTGTGTAAATGG GCAGTCTC, R-  
CCTCGTCTACCAAAGCGATA;

[0078] Exon 7 (part1): F-GCTTTTATTTCTACAT GTCC, R-  
GCTGTGCCAATCCTGAGATC;

[0079] Exon 7 (part2): F-GAGCATCAGCTATAACTG G. R-  
TAATGGATTCTCTGAACCTAC;

[0080] Exon 8 (part1): F-TTGAAATGTATGCCTGTAAAC, R-  
TTTCCATGCCTCAACATAAGG;

[0081] Exon 8 (part2): F-GTTTTTACCACAGCTGTGCC, R-  
ATACCTTAAGATCAATAGGATC.

[0082] 25 ml PCR reactions contained 2.5 mM dNTP, 1mM  
MgCl<sub>2</sub>, 1.25 unit of Taq polymerase (Gibco BRL) and 25 ng  
DNA. Amplification was done using 10' initial  
denaturation at 94°C, 35 cycles of 30'' at 94°C, 30'' at

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55°C and 1' 72°C with a final extension of 5' at 72°C. PCR products were purified using the Millipore Multiscreen<sup>®</sup>-PCR Plates, and their approximate concentrations estimated using a DNA size standard (BRL). Mutation detection was done by direct sequencing of both strands of PCR products on an ABI377 automated sequencer using BigDye Terminator chemistry (Applied Biosystems). Analysis was done with Factura software and Sequence Navigator (Applied Biosystems) for heterozygous base calls and sequence alignment.

[0083]

Testing of the base change in exon 5 in remaining family members and controls was done using Allele Specific Oligonucleotide Hybridization (ASO). PCR products containing exon 5 were amplified as described under sequencing. PCR products were blotted onto positively charged membranes. The blots were hybridized at 37°C for 45' with either the normal (TATTGCA~~A~~AATTGGC) or mutated sequence (TATTGCACATTGGC). Filters were washed until a final stringency of 0.3 x SSC/0.1% SDS was obtained for 15' at 37°C.

#### 4. Protein structure prediction and amino acid alignment

[0084]

Sequences were found by homology searches of the

human protein sequence to the non-redundant database using the BLAST algorithm. Implementing the Clustal-W algorithm the program Vector NTI aligned the sequences.

5 [0085] The protein structure was predicted using PEPTIDE STRUCTURE and PEPPILOT programs from Genetics Computer Groups (GCG) to obtain hydropathy plots. Transmembrane domains were identified and their topography predicted using TopPred, PHDhtm, SOSUI, HMMTOP, TMHMM, Tmpred programs. These programs predicted 9 or 10 transmembrane domains and all predicted the N144H mutation to be in a transmembrane domain.

10  
15  
20 [0086] Northern blots containing multiple adult human tissues (2 mg of polyA mRNA per lane) (Clontech), and multiple adult human digestive system tissues (1 mg of polyA mRNA per lane) (Clontech) and a Northern blot containing human monocytes and lymphoblast cells (10 mg total RNA) were hybridised with a genomic 431 bp. PCR product, from within exon 7 of SLC11A3, using ExpressHybä hybridisation solution (Clontech) according to the manufacturers instructions. Labeling of the probe was done using the Strip-EZ DNAä kit (AMBION).

### C. Applications

#### 1. HH Screening

25 [0087] With knowledge of the primary mutation of the

mutated SLC11A3 gene as disclosed herein, screening for presymptomatic heterozygotes, including prenatal diagnosis, and screening for heterozygotes can be readily carried out.

5 [0088] There are at least four levels at which the  
diagnostic information from the mutated SLC11A3 gene can  
be used. The first is to assist in the medical diagnosis  
of a symptomatic patient. In this application, a patient  
with a high index of suspicion for being affected with HH  
could be tested with the gene-based diagnostic. A  
positive result would show that the individual was  
heterozygous for the common HH mutation. This would  
provide a rapid and non-invasive confirmation that the  
individual corresponded to the fraction of the population  
heterozygous for this mutation. Such a result would help  
rule out other causes of iron overload in that  
individual.

[0089] The second level of application would be in first  
degree relatives of newly diagnosed probands. Currently  
recommended medical practice is to screen all such first  
degree relatives, as they are at a higher risk for  
disease and, if identified, could benefit from  
therapeutic intervention.



[0090]

The third level of screening would be in individuals afflicted with diseases that are known to be sequelae of HH disease. Such diseases include cirrhosis of the liver, diabetes, arthritis, reproductive dysfunction, and heart disease. It has been estimated, for example, that as many as 1% of individuals with diabetes may be so afflicted because of HH disease. In addition, other conditions such as sporadic porphyria cutanea tarda can be screened for using a mutated SLC11A3 gene diagnosis. When secondary to HH disease, some of the pathology of these diseases can be reversed upon phlebotomy therapy. Furthermore, it has been disclosed that the potential for hemochromatosis interferes with the effectiveness of interferon treatment of hepatitis C (Bacon, B. Abstracts of the Fifth Conference of the International Association for the Study of Disorders of Iron Metabolism 15-16 1995)). Therefore, it will be beneficial to perform screening with gene-based diagnostics in these disease populations.

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20 [0091]

The fourth level of screening is to screen the general population for heterozygotes. Several cost-benefit analyses have suggested that there is value in such screenings for the identification of presymptomatic

individuals. Once identified, such individuals could be targeted for preventative phlebotomy or treatment with the therapeutic compositions of the invention.

5 2. Nucleic Acid Based Screening

[0092] Individuals carrying mutations in the SLC11A3 gene may be detected at either the DNA, the RNA, or the protein level using a variety of techniques that are well known in the art. The genomic DNA used for the diagnosis may be obtained from body cells, such as those present in peripheral blood, urine, saliva, bucca, surgical specimen, and autopsy specimens. The DNA may be used directly or may be amplified enzymatically in vitro through use of PCR (Saiki et al. Science 239:487-491 (1988)) or other in vitro amplification methods such as the ligase chain reaction (LCR) (Wu and Wallace Genomics 4:560-569 (1989)), strand displacement amplification (SDA) (Walker et al. Proc. Natl. Acad. Sci. U.S.A. 89:392-396 (1992)), self-sustained sequence replication (3SR) (Fahy et al. PCR Methods Appl. 1:25-33 (1992)), prior to mutation analysis. The methodology for preparing nucleic acids in a form that is suitable for mutation detection is well known in the art.

[0093]

The detection of mutations in specific DNA sequences, such as in the SLC11A3 gene, can be accomplished by a variety of methods including, but not limited to, restriction-fragment-length-polymorphism detection based on allele-specific restriction-endonuclease cleavage (Kan and Dozy Lancet ii:910-912 (1978)), hybridization with allele-specific oligonucleotide probes (Wallace et al. Nucl Acids Res 6:3543-3557 (1978)), including immobilized oligonucleotides (Saiki et al. Proc. Natl. Acad. Sci. U.S.A. 86:6230-6234 (1989)) or oligonucleotide arrays (Maskos and Southern Nucl Acids Res 21:2269-2270 (1993)), allele-specific PCR (Newton et al. Nucl Acids Res 17:2503-2516 (1989)), mismatch-repair detection (MRD) (Faham and Cox Genome Res 5:474-482 (1995)), binding of MutS protein (Wagner et al. Nucl Acids Res 23:3944-3948 (1995)), denaturing-gradient gel electrophoresis (DGGE) (Fisher and Lerman et al. Proc. Natl. Acad. Sci. U.S.A. 80:1579-1583 (1983)), single-strand-conformation-polymorphism detection (Orita et al. Genomics 5:874-879 (1983)), RNAase cleavage at mismatched base-pairs (Myers et al. Science 230:1242 (1985)), chemical (Cotton et al. Proc. Natl. Acad. Sci. U.S.A. 85:4397-4401 (1988)) or

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enzymatic (Youil et al. Proc. Natl. Acad. Sci. U.S.A. 92:87-91 (1995)) cleavage of heteroduplex DNA, methods based on allele specific primer extension (Syvanen et al. Genomics 8:684-692 (1990)), genetic bit analysis (GBA) (Nikiforov et al. Nucl Acids Res 22:4167-4175 (1994)), the oligonucleotide-ligation assay (OLA) (Landegren et al. Science 241:1077 (1988)), the allele-specific ligation chain reaction (LCR) (Barrany Proc. Natl. Acad. Sci. U.S.A. 88:189-193 (1991)), gap-LCR (Abravaya et al. Nucl Acids Res 23:675-682 (1995)), and radioactive and/or fluorescent DNA sequencing using standard procedures well known in the art.

[0094] In addition to the genotype described above, single base-pair polymorphisms can also be used to assist in the identification of an individual whose genome contains the SLC11A3 mutation using procedures well known in the art.

### 3. Antibodies

[0095] As mentioned above, antibodies can also be used for the screening of the presence of the mutant SLC11A3, and the protein products therefrom. In addition, antibodies are useful in a variety of other contexts in accordance with the present invention. As will be appreciated,

antibodies can be raised against various epitopes of the mutant SLC11A3 protein. Such antibodies can be utilized for the diagnosis of HH and, in certain applications, targeting of affected tissues.

5 [0096] Thus, in accordance with another aspect of the present invention a kit is provided that is suitable for use in screening and assaying for the presence of the mutated SLC11A3 gene by an immunoassay through use of an antibody which specifically binds to a gene product of the mutated SLC11A3 gene in combination with a reagent for detecting the binding of the antibody to the gene product.

10 [0097] Antibodies raised in accordance with the invention can also be utilized to provide extensive information on the characteristics of the protein and of the disease process and other valuable information by process well known in the art.

#### 4. Peptide Presentation Assay

20 [0098] Peptide presentation can be measured through use of a number of well known techniques. One method is to express the SLC11A3 gene product on the surface of mammalian cells. Thereafter, the SLC11A3 gene product

can be purified from the cell surface analyzed for peptide binding, through, for example, high performance liquid chromatography (HPLC) after elution. Amino acid sequences of any bound peptides can be determined through conventional sequencing techniques.

[0099] Another technique to analyze peptide presentation is to express the SLC11A3 mutated gene product on a cell that does not conventionally possess peptide presentation activity. In such a system, MHC Class I molecules are expressed on the cell surface "empty" (i.e., without any bound peptide). Thereafter, through the addition of a particular peptide to the system, the binding of the particular peptide to the empty Class I molecule can be measured. A similar assay can be utilized in connection with the SLC11A3 mutated gene products.

[0100] The present inventive subject matter also contemplates kits for use in determining iron overload or iron underload potential, specifically kits identifying HH or anaemia in a patient. Preferred kit components generally are those that detect the agents discussed herein.

[0101] The kits may include one or more control results, such as reference slides of cyto-chemical results

characteristic of high and low iron overload or underload potential, or reference slides of in situ hybridization results characteristic in the same regard. Preferably kits will include a control series for interpreting results. The control series may be in the form of one or more photographs or may be depicted in other ways, including written descriptions.

[0102] A preferred kit contemplates treating HH, comprising a catalytically active fragment of an amino acid sequence as shown in SEQ ID No. 2, SEQ ID NO. 3 or SEQ ID NO. 4 in a pharmaceutically acceptable carrier and a device for delivery of the catalytically active fragment to the cells in need thereof, wherein the catalytically active fragment, the carrier and the device are packaged in a container.

[0103] The present invention also provides a kit for detecting or identifying HH or anaemia comprising: a) means for collecting a sample of breast epithelial cells; b) means for detecting an inhibitor of phospholipase A.sub.2, such as uteroglobin, in the sample of breast epithelial cells; and c) at least one reference which correlates to the amount of inhibitor of phospholipase A.sub.2 in normal breast epithelial cells or in

metastatic breast cancer cells, wherein differential amounts of the inhibitor of phospholipase A.sub.2 between the sample and the reference detects or identifies metastatic breast cancer.

5 [0104] Another preferred embodiment is a kit for detecting or identifying HH or anaemia, comprising: a) means for collecting a sample of DNA; and b) means for detecting a catalytically active fragment of an DNA sequence as shown in SEQ ID NO. 1, in the DNA sample.

10 5. Therapeutics

10 [0105] Identification of the SLC11A3 mutated gene and its gene product also has therapeutic implications. Envisioned are pharmacological, protein replacement, antibody therapy, and gene therapy approaches. In addition, the development of animal models useful for developing therapies and for understanding the molecular mechanisms of HH disease are envisioned. Under a pharmaceutical approach, drugs which circumvent or overcome the mutated SLC11A3 gene function may be designed. Accordingly, agents or drugs which are designed to interact with different aspects of the SLC11A3 protein structure or function or which mimic the



SLC11A3 protein interaction with other molecules can modulate SLC11A3 gene function.

[0106] Alternatively, a drug might bind to a specific functional residue(s) thereby, increasing or decreasing the affinity for ligand, substrate or cofactor. The assay for such a compound would be to promote or inhibit an interaction between the HH protein and similar molecules.

[0107] The present invention also relates to the use of polypeptide or protein replacement therapy for those individuals determined to have a mutated SLC11A3 gene. Treatment of HH disease could be performed by replacing the mutated SLC11A3 protein with normal protein or its functional equivalent in therapeutic amounts.

[0108] The present invention also relates to a pharmaceutical composition comprising: (i) a catalytically active fragment of an amino acid sequence as shown in SEQ ID No. 2, SEQ ID NO. 3 or SEQ ID NO. 4 and (ii) a pharmaceutically acceptable carrier.

[0109] The above discussion relating to the utility and administration of the compounds of the present invention also applies to the pharmaceutical compositions of the present invention.

[0110]           The term "pharmaceutically acceptable carrier" as used herein refers to any carrier, diluent, excipient, suspending agent, lubricating agent, adjuvant, vehicle, delivery system, emulsifier, disintegrant, absorbant, preservative, surfactant, colorant, flavorant, or sweetener.

[0111]           For these purposes the compounds of the present invention may be administered orally, parenterally, by inhalation spray, topically, rectally, nasally, buccally, vaginally or via an implanted reservoir in dosage formulations containing conventional non-toxic pharmaceutically-acceptable carriers, adjuvants and vehicles. The term parenteral as used herein includes subcutaneous, intravenous, intramuscular, intraperitoneally, intrathecally, intraventricularly, intrasternal and intracranial injection or infusion techniques.

[0112]           For oral administration, the compounds of the present invention may be provided in any suitable dosage form known in the art. For example, the compositions may be incorporated into tablets, powders, granules, beads, chewable lozenges, capsules, liquids, aqueous suspensions or solutions, or similar dosage forms, using conventional

equipment and techniques known in the art. Tablet dosage forms are preferred. Tablets may contain carriers such as lactose and corn starch, and/or lubricating agents such as magnesium stearate. Capsules may contain diluents including lactose and dried corn starch. Aqueous suspensions may contain emulsifying and suspending agents combined with the active ingredient.

[0113] When preparing dosage form incorporating the compositions of the invention, the compounds may also be blended with conventional excipients such as binders, including gelatin, pregelatinized starch, and the like; lubricants, such as hydrogenated vegetable oil, stearic acid, and the like; diluents, such as lactose, mannose, and sucrose; disintegrants, such as carboxymethylcellulose and sodium starch glycolate; suspending agents, such as povidone, polyvinyl alcohol, and the like; absorbants, such as silicon dioxide; preservatives, such as methylparaben, propylparaben, and sodium benzoate; surfactants, such as sodium lauryl sulfate, polysorbate 80, and the like; colorants such as F.D. & C. dyes and lakes; flavorants; and sweeteners.

[0114] Compositions and methods of the invention also may utilize controlled release technology. Thus, for example,

the inventive compounds may be incorporated into a hydrophobic polymer matrix for controlled release over a period of days. Such controlled release films are well known to the art. Particularly preferred are transdermal delivery systems. Other examples of polymers commonly employed for this purpose that may be used in the present invention include nondegradable ethylene-vinyl acetate copolymer and degradable lactic acid-glycolic acid copolymers which may be used externally or internally. Certain hydrogels such as poly(hydroxyethylmethacrylate) or poly(vinylalcohol) also may be useful, but for shorter release cycles than the other polymer releases systems, such as those mentioned above.

[0115] To be effective therapeutically as central nervous system targets, the compounds of the present invention should readily penetrate the blood-brain barrier when peripherally administered. Compounds which cannot penetrate the blood-brain barrier can be effectively administered by an intraventricular route or other appropriate delivery system suitable for administration to the brain.

[0116] The compounds of the present invention may be administered in the form of sterile injectable

5 preparations, for example, as sterile injectable aqueous  
or oleaginous suspensions. These suspensions may be  
formulated according to techniques known in the art using  
suitable dispersing or wetting agents and suspending  
agents. The sterile injectable preparations may also be  
sterile injectable solutions or suspensions in non-toxic  
parenterally-acceptable diluents or solvents, for  
example, as solutions in 1,3-butanediol. Among the  
acceptable vehicles and solvents that may be employed are  
water, Ringer's solution and isotonic sodium chloride  
solution. In addition, sterile, fixed oils are  
conventionally employed as solvents or suspending  
mediums. For this purpose, any bland fixed oil may be  
employed including synthetic mono- or di-glycerides.  
Fatty acids such as oleic acid and its glyceride  
derivatives, including olive oil and castor oil,  
especially in their polyoxyethylated versions, are useful  
in the preparation of injectables. These oil solutions or  
suspensions may also contain long-chain alcohol diluents  
or dispersants.

[0117] The compounds of this invention may also be  
administered rectally in the form of suppositories. These  
compositions can be prepared by mixing the drug with a

suitable non-irritating excipient which is solid at room temperature, but liquid at rectal temperature and, therefore, will melt in the rectum to release the drug. Such materials include cocoa butter, beeswax and polyethylene glycols.

[0118] The compounds of this invention may also be administered topically, especially when the conditions addressed for treatment involve areas or organs readily accessible by topical application, including neurological disorders of the eye, the skin, or the lower intestinal tract. Suitable topical formulations are readily prepared for each of these areas.

[0119] For topical application to the eye, or ophthalmic use, the compounds can be formulated as micronized suspensions in isotonic, pH adjusted sterile saline, or, preferably, as solutions in isotonic, pH adjusted sterile saline, either with or without a preservative such as benzylalkonium chloride. Alternatively for the ophthalmic uses the compounds may be formulated in an ointment such as petrolatum.

[0120] For topical application to the skin, the compounds can be formulated in a suitable ointment containing the compound suspended or dissolved in, for example, a

5 mixture with one or more of the following: mineral oil,  
liquid petrolatum, white petrolatum, propylene glycol,  
polyoxyethylene polyoxypropylene compound, emulsifying  
wax and water. Alternatively, the compounds can be  
formulated in a suitable lotion or cream containing the  
active compound suspended or dissolved in, for example,  
a mixture of one or more of the following: mineral oil,  
sorbitan monostearate, polysorbate 60, cetyl esters wax,  
cetearyl alcohol, 2-octyldodecanol, benzyl alcohol and  
10 water.

[0121] Topical application for the lower intestinal tract  
an be effected in a rectal suppository formulation (see  
above) or in a suitable enema formulation.

15 [0122] Dosage levels on the order of about 0.1 mg to about  
10,000 mg of the active ingredient compound are useful in  
the treatment of the above conditions, with preferred  
levels of about 0.1 mg to about 1,000 mg. The amount of  
active ingredient that may be combined with the carrier  
materials to produce a single dosage form will vary  
20 depending upon the host treated and the particular mode  
of administration.

[0123] It is understood, however, that a specific dose  
level for any particular patient will depend upon a

variety of factors including the activity of the specific compound employed, the age, body weight, general health, sex, diet, time of administration, rate of excretion, drug combination, and the severity of the particular disease being treated and form of administration.

[0124] The compounds as referenced above can be administered with other drugs treating HH or anaemia. The dosage level of other drugs will depend upon factors and the effectiveness of the drug combination known to those skilled in the art.

[0125] Gene therapy utilizing recombinant DNA technology in vivo is also contemplated within the scope of the present invention. It is within the scope of the invention to deliver the normal form of the SLC11A3 gene into patient cells or vectors which will supply the patient with gene product. In gene therapy of HH disease, a normal version of the SLC11A3 gene is delivered to affected tissue(s) in a form and amount such that the correct gene is expressed and will prepare sufficient quantities of SLC11A3 protein to reverse the effects of the mutated SLC11A3 gene. Current approaches to gene therapy include viral vectors, cell-based delivery systems and delivery agents.

[0126] Further, ex vivo gene therapy could also be useful.



In *ex vivo* gene therapy, cells (either autologous or otherwise) are transfected with the normal SLC11A3 gene or a portion thereof and implanted or otherwise delivered into the patient. Such cells thereafter express the normal SLC11A3 gene product *in vivo* and would be expected to assist a patient with HH disease in avoiding iron overload normally associated with HH disease. *Ex vivo* gene therapy is described in U.S. Pat. No. 5,399,346 to Anderson et al., the disclosure of which is hereby incorporated by reference in its entirety. Conversely, *ex vivo* gene therapy in patients suffering from anemia would be expected to assist a patient by promoting iron uptake by introducing cells transfected with mutated SLC11A3.

[0127]

The invention being thus described, it will be obvious that the same may be varied in many ways. Such variations are not to be regarded as a departure from the spirit scope of the invention and all such modifications are intended to be included within the scope of the following claims.